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Improved GM1-Enzyme-Linked Immunosorbent Assay for Detection of *Escherichia coli* Heat-Labile Enterotoxin

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Previously described GM1 ganglioside enzyme-linked immunosorbent assays (GM1-ELISA) for the detection of *Escherichia coli* heat-labile enterotoxin (LT) showed sensitivity equal to the Y-1 adrenal cell assay when anti-LT (a reagent not commercially available) was used. However, when antitoxin to immunologically related (commercially available) cholera toxin was substituted, a marked loss in sensitivity occurred. We modified the GM1-ELISA that employed anti-cholera toxin to make it comparable in sensitivity to the Y-1 adrenal cell assay. When five media commonly used for LT production were compared, Mundell's Casamino Acids medium was shown to be significantly superior. Lincomycin (45 μg/ml) added to *E. coli* cultures significantly increased net optical densities in the GM1-ELISA, a direct measure of the amount of LT. Treatment of broth cultures or bacterial cell pellets with polymyxin B or extension of culture time to 48 h also significantly increased net optical density by allowing enhanced release of periplasmic LT. A major innovation involved the direct culture of *E. coli* strains in GM1-coated wells of microtiter plates followed by ELISA. This direct culture method GM1-ELISA (DCM-GM1-ELISA) saved not only assay time, but also materials and reagents. The net optical densities that result from this assay allow the test to be read visually without a spectrophotometer. Three independent observers read plates with *E. coli* tested by DCM-GM1-ELISA. Thirty-four of 35 adrenal cell-positive strains (97% sensitivity) and 30 of 30 LT-negative control *E. coli* strains (100% specificity) were identified by all three observers reading coded plates. The DCM-GM1-ELISA provides a simple, practical and efficient assay for LT for less sophisticated laboratories.

Enterotoxigenic *Escherichia coli* are major causative agents of travelers' diarrhea (11), infantile diarrhea in less-developed countries (1), and colibacillosis in neonatal herd animals (14, 22). Approximately one-half of enterotoxigenic strains of *E. coli* produce a heat-labile toxin (LT) either alone or in conjunction with a heat-stable toxin (ST) (17, 20, 21). Before 1977, assays used to detect LT required either animals (4, 5, 7, 8), tissue culture cells (6, 12, 19, 23), or special equipment and sterile environments (6, 12, 19, 23). Consequently, assays for the detection of LT had been restricted to research and reference laboratories. Since 1977, two methods for identification of LT have been described which are sensitive, relatively inexpensive, and simple to perform; these include the enzyme-linked immunosorbent assay (ELISA) (26) and the Biken test (13). Because the Biken test requires 3 to 5 days and works best with anti-LT sera (which is not commercially available) and because ELISA technology is so widespread, we undertook evaluation of the latter for identification of LT. Yolken et al. (26) first developed an ELISA for the detection of LT. Svennerholm et al. (24) described a helpful modification wherein the putative receptor for LT, GM1 ganglioside, was utilized as a capture molecule for LT, resulting in an assay comparable in sensitivity to the Y-1 adrenal cell assay. The drawback of this modification was the need for anti-LT serum. When Sack et al. (18) substituted (for LT antitoxin) immunologically related high-titer cholera antitoxin (prepared by immunizing guinea pigs with commercially available purified cholera toxin), the assay was less sensitive than the Y-1 adrenal cell assay. Following Sack's procedure and using Casamino Acids medium B, described by Evans et al. (8), we detected only 40% of adrenal cell-positive enterotoxigenic *E. coli* strains, thus confirming that the GM1-ELISA with cholera antitoxin was less sensitive than the Y-1 adrenal cell assay for identification of LT.

To circumvent the need for anti-LT, we investigated ways to enhance the sensitivity of the GM1-ELISA, while still utilizing cholera anti-

TABLE 1. Composition of five media widely used for LT production

Medium (reference)	Composition[a]						
	Casamino Acids (Difco) (g)	Yeast extract (Difco) (g)	NaCl (g)	K_2HPO_4 (g)	Glucose (g)	Trace salts solution (ml)[b]	Final pH[c]
Mundell's Casamino Acids (25)	20	6	2.5	8.71	2.5	1 (no. 2)	8.5
Biken (13)	20	10	2.5	15	5	0.5 (no. 1)	7.5
Sack's Casamino Acids with yeast extract (18)	30	3		0.5	2.0		8.0
Evan's Casamino Acids A (10)	20	6	2.5	8.71		1 (no. 3)	8.5
Evan's Casamino Acids B (8)	20	6	2.5	8.71		1 (no. 2)	8.5

[a] All media were made with 1 liter of distilled water.
[b] Solution no. 1, 5% $MgSO_4$, 0.5% $FeCl_3$, and 2% $CoCl_2 \cdot 6H_2O$; solution 2, 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$; solution no. 3, 5% $MgSO_4$, 5% $MnCl_2$, and 0.5% $FeCl_3$.
[c] The final pH was adjusted with NaOH.

toxin. These investigations included a comparison of culture media, studies of the effect of lincomycin in increasing LT production, and means of enhancing the release of toxin from the periplasmic space of *E. coli* cells.

MATERIALS AND METHODS

Strains of *E. coli*. Thirty-five LT-positive strains of *E. coli* from persons with diarrhea were confirmed by Y-1 adrenal cell assay (19). Thirty adrenal cell-negative strains included LT^-/ST^- enteropathogenic *E. coli* strains from patients with diarrhea, strains cultured from healthy Peace Corps volunteers, LT^-/ST^+ strains from patients with travelers' diarrhea, and a strain from a healthy American. In addition to strains from the Center for Vaccine Development collection, strains were kindly provided by Isis A. Mikhail, Naval Medical Research Unit, Cairo, Egypt; Hugo Mendoza, Santo Domingo, Dominican Republic; R. Bradley Sack, Baltimore City Hospitals, Md.; George Morris, the Centers for Disease Control, Atlanta, Ga.; and Bernard Rowe, Department of Enteric Pathogens, Central Public Health Laboratories, London, England. All strains were frozen in skim milk and stored at −70°C.

Bacterial cultivation. Five media were compared for their ability to support toxin production. Each strain was plated onto Trypticase soy agar containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.), and after overnight incubation at 37°C, a loopful of bacteria was transferred to 5 ml of medium in a 50-ml Erlenmeyer flask. All media were prepared the same day, and the same lots of Casamino Acids and yeast extract were used throughout the study. The composition of each medium is seen in Table 1. All strains were grown aerobically for 18 h at 37°C while shaking on a Lab-Line Junior Orbit rotary shaker (150 rpm). After incubation, the cultures were centrifuged at 8,000 × *g* for 15 min, and the supernatants were assayed that day or stored at 4°C for not longer than 2 days before testing.

Antibiotic treatment. Lincomycin (The Upjohn Co., Kalamazoo, Mich.) was added to media to a concentration of 45 μg/ml to assess its effect on toxin induction (3, 15). Preliminary studies showed that higher concentrations (70 and 90 μg/ml) of lincomycin inhibited growth of some enterotoxigenic *E. coli* strains; therefore, 45 μg/ml was chosen.

In assessing the effect of polymyxin B sulfate (PMB) (Pfizer, Inc., New York, N.Y.) on release of toxin from the periplasmic space, cultures were treated in two ways: (i) PMB was added to a bacterial cell pellet (25) to a concentration of 10,000 U/ml and (ii) PMB was added to a suspension of bacterial cells to a concentration of 10,000 U/ml. After shaking for 30 min at 37°C, the PMB-treated bacterial cells were centrifuged at 8,000 × *g* for 15 min, and the supernatants were assayed for LT.

Hyperimmune rabbit antiserum to cholera toxin. Two adult New Zealand albino rabbits (Bunnyville Farms, Littletown, Pa.) weighing 3.5 kg, were injected subcutaneously every other day for 1 week and then once weekly for 6 weeks with 100-μg doses of purified enterotoxin from *Vibrio cholerae* (Schwarz/Mann, Orangeburg, N.Y.) in Freund incomplete adjuvant. After a 1-week rest, the rabbits were bled by cardiac puncture, and the sera were separated, pooled, and stored at −70°C. The working dilution of the antisera was determined by a checkerboard titration within the specific assay system and was found to be 1:3,200.

Conjugate. Alkaline phosphatase-labeled, affinity-purified goat antibody to rabbit IgG (heavy and light chains) was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, Md.; lot no. CL21).

GM1-ELISA method. Alternate wells of a polystyrene 96-well microtiter plate (Costar, Cambridge, Mass.) were coated overnight at room temperature (20 to 22°C) with 100 μl of GM1 ganglioside (1.0 μg/ml) (Supelco, Bellafonte, Pa.) in 0.1 M phosphate-buffered saline containing 0.01 M sodium phosphates and 0.1 M NaCl (pH 7.2) (PBS). The remaining wells were incubated with PBS and served as background control

wells for nonspecific binding of all subsequent reagents. The plates were then washed three times with PBS containing 1% polyoxyethylene (21) sorbitan monolaurate (Tween 20; J. T. Baker Chemical Co., Phillipsburgh, N.J.). Plates are then used or can be stored at −70°C for up to 2 months. Next, the remaining unbound reaction sites were blocked by the addition of 200 μl of heat-inactivated (56°C for 30 min) 5% fetal calf serum in PBS to all wells for 1 h at 37°C. The wells were emptied and washed, as above, after which 100 μl of test filtrates was added to the test and background control wells for a 90-min incubation at 37°C. After washing, 100 μl of rabbit serum (anti-cholera toxin; 1:3,200) was added to all wells and incubated for 1 h at 37°C. After another washing procedure, 100 μl of alkaline phosphatase-labeled goat anti-rabbit IgG serum (1:200) was incubated in all wells under the same conditions. After a final washing procedure, 100 μl of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) diluted to 1 mg/ml in 10% DEAE buffer (pH 9.8) was added to all wells and incubated for 45 min at 37°C. The reaction was then stopped with the addition of 25 μl of 3 N NaOH. Optical densities (OD) were measured with a spectrophotometer at a wavelength of 400 nm. The net OD was calculated by subtracting the OD of the background control wells from that of the corresponding test well. Plates were also read visually, and a positive or negative result was recorded.

Direct culture method-GM1-ELISA (DCM-GM1-ELISA). Wells of a microtiter plate were coated overnight with GM1 ganglioside as previously described. After the wells were washed three times with PBS and incubated with fetal calf serum for 1 h, followed by another washing, 200 μl of Mundell's medium was added, and the test strains were inoculated directly into the wells, using sterile toothpicks. After 24 h of incubation on a rotary shaking platform (150 rpm) at 37°C, plates were washed and the assay completed as previously described.

Sensitivity of GM1-ELISA. To determine the sensitivity of the GM1-ELISA, we tested various dilutions of cholera toxin and *E. coli* human LT. Commercially purified cholera toxin was obtained from Schwartz/Mann, and purified LT was kindly supplied by Richard Finkelstein (University of Missouri School of Medicine, Columbia). Each toxin preparation was serially diluted twofold in PBS to yield concentrations from 400 to 0.1 ng/ml. A sample of each dilution was added to a set of test wells of a microtiter plate, and the assay was carried out as previously described.

RESULTS

Sensitivity of GM1-ELISA. The titration curves for cholera toxin and *E. coli* LT are shown in Fig. 1A. The least amount of toxin detected by the GM1-ELISA was 0.6 ng/ml for cholera toxin and 7.0 ng/ml for purified *E. coli* LT (Fig. 1B).

Effects of media on toxin production. To determine the effects of the medium on toxin production, 10 known LT-positive and 10 negative control strains were grown in five media which are widely used for bacterial toxin production

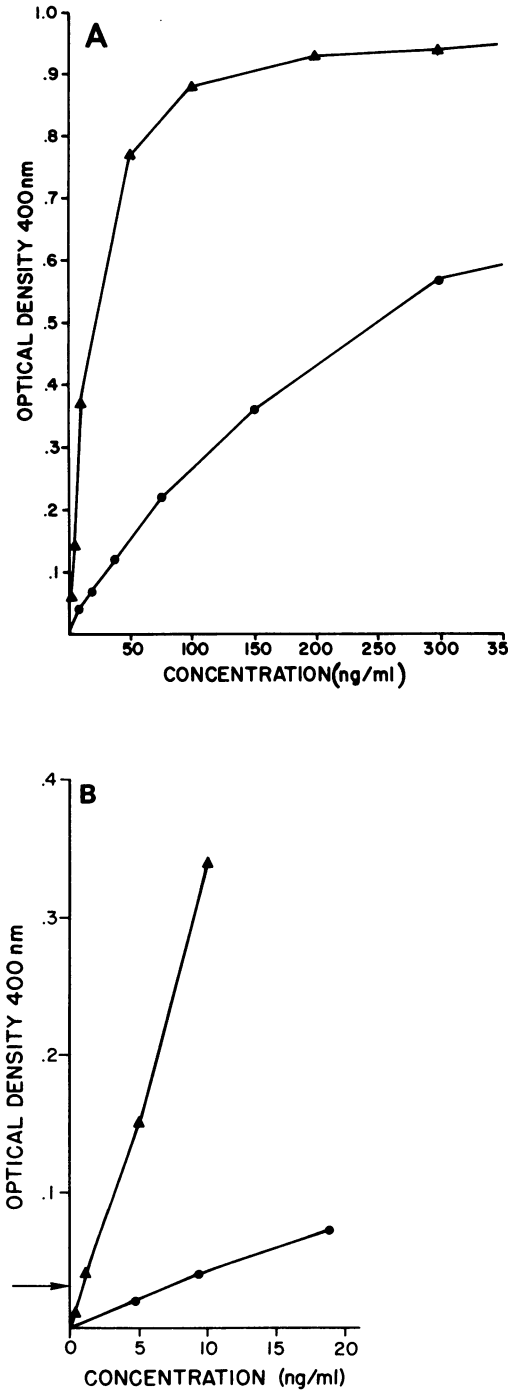

FIG. 1. (A) Titration curves of purified cholera toxin (▲) and *E. coli* LT (●) in GM1-ELISA. (B) Titration curves of purified cholera toxin (▲) and *E. coli* LT (●) showing the lower concentrations of toxin. The arrow indicates the minimum OD (0.04) which is still considered positive for toxin production in the GM1-ELISA when the ELISA plates are read with a spectrophotometer.

TABLE 2. Comparison of effects of five media with or without lincomycin on LT production by 20 strains of *E. coli* assayed by GM1-ELISA[a]

Medium	Lincomycin (μg/ml)	Adrenal cell-positive strains (n = 10)		
		Group mean net OD ± SEM	Mean of positives ± SEM	No. of positives with ODs > 0.10
Mundell's Casamino Acids	0	0.27 ± 0.03	0.27 ± 0.03	10
	45	0.44 ± 0.02	0.49 ± 0.03	10
Biken	0	0.18 ± 0.03	0.22 ± 0.03	6
	45	0.29 ± 0.03	0.35 ± 0.03	8
Sack's Casamino Acids	0	0.19 ± 0.03	0.24 ± 0.03	7
	45	0.36 ± 0.03	0.44 ± 0.03	8
Evan's Casamino Acids A	0	0.06 ± 0.02	0.14 ± 0.02	1
	45	0.31 ± 0.03	0.37 ± 0.03	8
Evan's Casamino Acids B	0	0.09 ± 0.02	0.17 ± 0.03	4
	45	0.29 ± 0.03	0.31 ± 0.02	9

[a] All 10 adrenal cell-negative strains tested had group mean net ODs ± standard error of the mean of 0.01 ± 0.00, and there were no positives among these strains with ODs > 0.10.

(Table 1). The cultures were centrifuged at 8,000 × g, and their supernatants were assayed for LT. The resultant net ODs are compared in Table 2. None of the negative control strains grown in any of the five media yielded a net OD > 0.03, and the mean net OD of these strains when grown in any medium was 0.01.

In the light of the net OD values obtained with the 10 negative control strains in this initial experiment, a net OD of 0.10 or greater was arbitrarily selected as the cutoff for evidence of LT, i.e., a positive test. Using this criterion, there was significant variation in the ability of the various media (in the absence of lincomycin) to support production of LT. With the best medium tested (Mundell's Casamino Acids medium), evidence of LT production was detected for all 10 adrenal cell-positive strains versus only 1 of 10 for Evans Casamino Acids A medium (P < 0.001) and 4 of 10 for Evans Casamino Acids B medium (P < 0.01). Among the 10 known LT-positive strains grown in the absence of lincomycin, the net ODs ranged from 0.02 to 0.62. Depending upon the growth medium, the mean net OD for the 10 strains ranged from 0.06 to 0.27. Casamino Acids medium described by Mundell was clearly the best medium tested, producing a mean net OD of 0.27. This medium was used for all future studies.

Effects of lincomycin. Preliminary experiments to assess the effects of lincomycin on LT production in the GM1-ELISA were carried out in conjunction with the experiments comparing various media. The 10 negative control strains and the 10 known LT-positive strains were concomitantly grown in the presence of 45 μg of lincomycin per ml. The results are also shown in Table 2. The addition of lincomycin to the various media significantly increased the mean net OD of the 10 strains (P < 0.001). It also increased the percentage of LT-positive strains (i.e., those having a net OD ≥ 0.10) but had no effect on the net OD of the 10 negative control supernatants. Since lincomycin so significantly enhanced toxin production, it was added to the media in all subsequent experiments.

PMB treatment. (i) PMB treatment of bacterial pellets. Cerny and Teuber (2) reported that PMB treatment of bacterial cells caused release of periplasmic proteins. To investigate the effect of PMB on enhancing toxin release, the cells in two samples of the same bacterial cell suspension were pelleted. One pellet was treated with PMB, the other with PBS. The amounts of toxin released from these pellets were compared with the amount in the non-PMB-treated supernatant. The pellets of the bacterial cells treated with PMB resulted in a mean net OD on ELISA of 0.37, which was significantly higher than that of similarly PBS-treated pellets, i.e., 0.13 (P < 0.001). The mean net OD of the PMB-treated pellet, however, was not higher than the mean net OD of the supernatants, 0.37 (Table 3).

(ii) PMB treatment of bacterial cultures. The above-described method of PMB treatment, which involves pelleting of bacterial cells, PMB treatment, and recentrifugation, is cumbersome, time consuming, and does not raise net OD above that found when the supernatant is tested. For these reasons, as well as to combine the

TABLE 3. Comparison of amount of LT in untreated supernatants, bacterial pellets, PMB-treated bacterial pellets, PMB-treated cell cultures, and 48-h cultures of 65 strains of *E. coli* grown in the presence of lincomycin (45 μg/ml)

Test materials	Mean net OD (±SEM) of:	
	35 adrenal cell-positive strains	30 adrenal cell-negative strains
Untreated supernatant	0.37 ± 0.04	0.01 ± 0.00
Pellet + PBS	0.13 ± 0.03	0.01 ± 0.00
Pellet + PMB	0.37 ± 0.04	0.01 ± 0.00
Cell culture + PMB	0.50 ± 0.04	0.01 ± 0.00
48-h growth	0.54 ± 0.02	0.01 ± 0.00

toxin present in the supernatant with the additional toxin released upon PMB treatment of cells, PMB was added directly to bacterial cultures. Direct treatment of bacterial cultures with PMB resulted in detection of a significantly greater amount of toxin than was found in the untreated supernatant (paired *t* test, $P < 0.001$) (Table 3).

(iii) Prolongation of culture time to promote LT release. PMB was effective in causing release of LT from the periplasmic space of *E. coli* grown in the presence of lincomycin. However, since PMB adds an additional expense and may not be readily available in less-developed countries, the effect of extended culture time (48 h) was explored as an alternative to enhance LT release. In theory, a prolonged growth phase should result in release of LT from disrupted, senescent cells. When *E. coli* was cultured in the presence of lincomycin, the mean net OD of 48-h culture supernatants (0.54) was significantly higher than that of the corresponding 18-h supernatants (0.37) (paired *t* test, $P < 0.001$) and was almost identical to the mean net OD of the PMB-treated whole cell culture (0.50) (Table 3). Since the mean net OD of the GM1-ELISA-tested strains, grown in the presence of lincomycin, was almost identical whether the cells were PMB treated or grown for 48 h, either method may be used. Minimization of delay and the availability of PMB in our laboratory influenced the choice of PMB treatment over the 48-h growth method.

The mean net OD of the 30 adrenal cell-negative strains remained consistently low when examined under any of the above test conditions, i.e., lincomycin, PMB, or growth for 48 h (Table 3). The mean net OD of the 30 adrenal cell-negative strains tested was 0.01 ± 0.01 (mean ± standard deviation). Therefore, a filtrate was statistically determined to be positive for toxin if its net OD was greater than the mean net OD of the negative population plus 3 standard deviations (i.e., >0.04).

DCM-GM1-ELISA. To lower the assay cost, to save time, labor, and materials, and to further adapt the GM1-ELISA for use in clinical laboratories, we undertook the simplification of the assay by culturing *E. coli* strains directly in situ in the same GM1-coated microtiter wells in which the ELISA would be carried out. The wells of a microtiter plate were coated with GM1 ganglioside, filled with medium and inoculated with the test strains. A total of 65 strains were tested by the DCM, comparing the effects of lincomycin, PMB, and growth of bacteria for 48 h. The results obtained with this DCM-GM1-ELISA are seen in Table 4.

The mean net OD of *E. coli* grown for 24 h in the absence of lincomycin and PMB was 0.15; extension of culture time to 48 h did not significantly increase the mean net OD (0.19) (Table 4). In contrast, 24 h of growth in the presence of lincomycin significantly increased the mean net OD (0.32) ($P < 0.001$). Addition of PMB to 24-h lincomycin-grown cultures did not further enhance the mean net OD (0.33), nor did extension of growth time to 48 h (0.31). None of the manipulations altered the net OD of the nontoxigenic negative control strains.

Visual reading of GM1-ELISA and DCM-GM1-ELISA. Visual determination of the assay results, thereby eliminating the need for a spectrophotometer, would greatly enhance the utility of the GM1-ELISA for field laboratories. Seventy microtiter plates (2,100 pairs of test wells) comprising both flask-grown and in situ DCM were read both spectrophotometrically and visually. Independent visual readings without knowledge of the contents of the wells were made by three separate individuals. If the amount of color in the GM1-coated test well was perceived to be greater than in the background well, a positive result was recorded; a negative visual result was recorded when no color difference was detected.

Of 895 wells with a net OD of ≤0.04, 855 were

TABLE 4. Effects of lincomycin (45 μg/ml), PMB, and 48-h growth on LT production by 35 adrenal cell-positive strains, as determined by DCM-GM1-ELISA

Culture conditions	No. of positive ODs > 0.04	Group mean net OD ± SEM
24 h		
Untreated	25	0.15 ± 0.02
PMB	26	0.16 ± 0.03
Lincomycin	34	0.32 ± 0.03
Lincomycin + PMB	34	0.33 ± 0.03
48 h		
Untreated	32	0.19 ± 0.02
Lincomycin	34	0.31 ± 0.02

TABLE 5. Comparison of visual and spectrophotometer reading of GM1-ELISA and DCM-GM1-ELISA for detection of LT in LT-positive and negative control *E. coli* strains

Test	*n*	No. (%) identified as LT positive	
		Visual	Spectrophotometer
GM1-ELISA[a]			
LT-positive strains[b]	35	35 (100)	35 (100)
LT-negative strains[b]	30	0	0
DCM-GM1-ELISA[c]			
LT-positive strains	35	34 (97)	34 (97)
LT-negative strains	30	0	0

[a] For modification with lincomycin and PMB see the text.

[b] Based on results of Y-1 adrenal cell assay.

[c] Modified ELISA in which *E. coli* strains are cultured in situ with lincomycin in the same GM1-coated microtiter plate wells in which the ELISA will be performed. See text for details.

detected visually as negative (96%), whereas 665 of 665 (100%) wells with a net OD $\geq$ 0.16 were recorded visually as positive, with complete agreement among all three observers. In contrast, both intraobserver and interobserver variations occurred in visual reading of wells with net ODs between 0.05 and 0.15. In practical terms, however, this does not present a problem since the modifications of the GM1-ELISA described in this report assure that the net OD of all 35 adrenal cell-positive strains grown in flasks and 34 of the 35 grown directly in wells of microtiter plates (DCM-GM1-ELISA) can be detected by the naked eye; i.e., all had net ODs $\geq$ 0.16 (Table 5). The one strain that was not detected as LT positive when tested by the DCM-GM1-ELISA had a net OD of 0.02 (Table 5); it was detected as adrenal cell positive only when cultured in flasks. Thirty adrenal cell negatives were tested by the DCM, and none were detected as positive when read visually or with a spectrophotometer.

DISCUSSION

Although LT-producing *E. coli* have been recognized as human enteropathogens for more than a decade, systematic study of their role in infant diarrhea in less-developed countries and in travelers' diarrhea has been limited by the shortcomings of current assays for LT. To be applicable for widespread use in less-developed countries, an assay for LT should be practical, rapid, technically simple, repeatable, sensitive, and specific, should use commercially available reagents, and should not require expensive equipment. Because of the increasing use of in ELISA techniques worldwide and the advantages inherent in ELISA (sensitivity, specificity, repeatability, economy, and ability to process large numbers of specimens), we undertook to modify and improve previously described GM1-ELISA methods to enhance sensitivity, improve economy, and augment simplicity. Since the Y-1 adrenal cell assay is generally accepted as the "gold standard" of sensitivity and specificity, we undertook to develop an ELISA that would be comparable in sensitivity to the Y-1 adrenal cell assay, while offering all the other abovementioned advantages. The GM1-ELISA method described by Svennerholm and Holmgren (24) is equivalent in sensitivity to the Y-1 adrenal cell assay, but its requirement for LT antitoxin, which is not available commercially, puts it beyond reach of most laboratories. Sack et al. (18) attempted to overcome this drawback by utilizing antitoxin to immunologically related cholera toxin. The result was an unacceptable loss in sensitivity. The series of modifications that we developed resulted in sensitivity equivalent to the Y-1 adrenal cell assay with use of cholera antitoxin.

Two critical steps in perfecting our assay involved identifying the optimal culture medium and assessing ways to enhance LT production and release. Ours was the first attempt to compare the various media described in the literature for production of LT in relation to detection of LT by ELISA. To our surprise, the five media varied significantly in their ability to support LT production as detected by ELISA (Tables 1 and 2). The Casamino Acids medium described by Mundell et al. (16) was clearly the most superior medium, and was used in all further studies. A more detailed study of the nutritional requirements for toxin production might lead to further enhancement of the amount of toxin elaborated.

It was also found that with each of the five media examined the addition of lincomycin (45 μg/ml) significantly increased the amount of LT produced as detected by ELISA (Tables 2 and 4). The exact mechanism of its effect on whole cells is not yet understood. However, it is known that lincomycin inhibits protein synthesis by blocking the peptidyl transferase function of the 50S ribosomal units (15). Levner et al. (15) postulated that toxin production may normally be limited by a protein whose synthesis may be more sensitive to inhibition by lincomycin than is the toxin protein.

After showing the value of lincomycin in increasing the production of LT in *E. coli* grown in flasks, we next attempted to augment release of LT from the periplasmic space of *E. coli* bacterial cells. Evans et al. (9) have reported that LT is rapidly released from *E. coli* bacterial pellets upon PMB treatment, indicating that LT is in part a cell-associated toxin. We found that

PMB indeed enhanced release of LT from bacterial pellets. A more practical observation was that the direct treatment of lincomycin-containing flask cultures with PMB notably increased the amount of toxin released into the supernatant; this was documented by significantly increased OD in the supernatants over those treated with PBS. As an alternative to PMB one can obtain increased release of LT into the supernatant by incubating the flask cultures for 48 h. Although this lengthens the time of the assay it precludes the use of PMB.

The DCM-GM1-ELISA, which involves growing test cultures directly in GM1 ganglioside-coated wells of a microtiter plate, represents an innovative breakthrough that has important implications for measurement of any bacterial antigen with GM1-ELISA. This innovation completely precludes the need to cultivate *E. coli* in flasks and to centrifuge cultures before performing ELISA in microtiter plates. The DCM-GM1-ELISA method saves assay time, materials, reagents, and labor. As with flask-grown cultures, lincomycin (45 μg/ml) doubled the net OD of strains tested by the DCM-GM1-ELISA method. PMB and extension of the incubation period from 24 to 48 h did not further increase the net OD of the test strains. PMB treatment or 48-h cultivation of *E. coli* appears to be unnecessary in the DCM-GM1-ELISA method, since LT released from toxigenic strains in the wells is probably being rapidly and reliably bound as it contacts the GM1 ganglioside receptor, thereby amplifying the immunoassay.

Since it is intended that this assay be utilized in laboratories in which microspectrophotometers are unavailable, visual interpretation of the results is compulsory. Our modifications of the GM1-ELISA, including use of an optimal medium with lincomycin and PMB, result in a situation in which 35 of 35 adrenal cell-positive *E. coli* strains grown in flasks yielded sufficient color to be read visually when tested by ELISA (Table 5); in every instance, the net OD exceeded 0.16, and there was complete agreement among three independent, unbiased visual observers unaware of the contents of the wells. Using the simple, economical DCM-GM1-ELISA modification, 34 of 35 adrenal cell-positive strains were correctly identified by visual reading of the plates (Table 5), yielding an acceptable test sensitivity of 97% and specificity of 100%.

The DCM-GM1-ELISA method described herein provides an attractive alternative to the Biken test for detection of LT-producing *E. coli* by less-sophisticated laboratories, particularly those in less-developed countries. The DCM-GM1-ELISA has certain distinct advantages over the Biken test. It is much quicker (2 days versus 3 to 5 days) and works extremely well with cholera antiserum (the Biken test requires LT antiserum for good sensitivity). It is anticipated that the DCM-GM1-ELISA method for detection of LT-producing *E. coli* will greatly enhance and expand study of the epidemiology of diarrhea due to this pathogen.

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